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Accumulation and Biodegradation of Dibutyl Phthalate in *Chlorella vulgaris*

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Phthalate esters (PAEs) as a class of synthetic organic compounds have a wide variety of industrial, agricultural and domestic applications, including construction, automobile, household products, apparel, toys, packaging, medical products, cosmetics, insect repellents, insecticide carriers, and propellants, etc. One of the most widely used PAEs is dibutyl phthalate (DBP). Large-scale and widespread production, combined with the fact that PAEs are not chemically bound to the polymeric matrix and are able to migrate from plastic products, provides the potential for this class of chemicals to enter into the aquatic environment. PAEs have been listed as 'priority pollutants' by the US EPA (Keith 1979). Recently, PAEs have been suspected to cause estrogenic effects in mammals including humans, and thus damage the reproductive systems (Environment Agency 1998).

Phytoplankton in many systems is the most abundant source of organic material in the water column, and thus plays an important role in determining the fate of persistent hydrophobic organic chemicals (HOCs) in aquatic. Bioaccumulation at the phytoplankton level can dominate the entry of HOCs into a food web. In addition, consumption of contaminated food, rather than direct uptake from the water, can be the major uptake route for top predators including fish. Freshwater and marine algae are the most important primary producer in the aquatic environment. It is meaningful to study the accumulation and degradation of HOCs by algae. Studies have shown that microalgae have the ability to degrade several organic and organometallic pollutants (Lee 1989; Klekner 1992). But only a few studies have focused on the degradation of PAEs by algae (Yan 1993; Huang 1999).

In this study, effects of initial DBP concentration and temperature on bioconcentration and biodegradation of DBP by fresh alga *Chlorella vulgaris* were investigated in a synthetic cultural medium. Bioconcentration and biodegradation of DBP added to lake water by fresh alga *Chlorella vulgaris* were also observed.

MATERIALS AND METHODS

DBP was purchased from Sigma (purity grade 99%). *Chlorella vulgaris* was obtained from the Institute of Hydrobiology, Academy of Science, China. The

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cultural medium consisted of $NaNO_3$, 0.25 g/L; NaCl, 0.025 g/L; $CaCl_2 \cdot 2H_2O$, 0.025 g/L; KH_2PO_4, 0.175 g/L; K_2HPO_4, 0.075 g/L; $MgSO_4 \cdot 7H_2O$, 0.075 g/L; EDTA·Na_2, 0.064 g/L; $FeSO_4 \cdot 7H_2O$, 0.005 g/L; H_2SO_4, 0.001 ml/L; H_3BO_3, 0.011 g/L; $ZnSO_4 \cdot 7H_2O$, 0.0088 g/L; $CuSO_4 \cdot 5H_2O$, 0.0016 g/L.

According to the standard method (American Public Health Association 1980) of algal bioassay for evaluating toxicity of toxic chemicals, the toxicity experiments of DBP to algae were conducted beforehand. Based on experimental data, the 96h-EC_{50} of DBP on inhibition of the growth of *Chlorella vulgaris* at 25℃ was calculated as 7.78 mg/L, and had no 96h-EC_{50} of inhibition in the range of its water solubility at 13℃ (Schwarz 1980; Schwarzenbach 2003).

For bioconcentration and biodegradation test in the culture medium, algae were grown in 250 mL flasks each containing 60 mL medium-DBP solution under sterilized condition, and the inoculated flasks were kept in a culture room at designed temperatures with the light intensity of 4000 ± 100 lux continuously. The initial density of algal cells, used to express algal biomass, was approximately 10^6 cell/mL. Controls were performed under identical conditions except without algae. DBP concentrations both in water and in algal cells were regularly determined. Experiments of bioconcentration and biodegradation for DBP were conducted in triplicate.

For investigating the matrix effect of a real aquatic environment, bioconcentration and biodegradation kinetics of DBP by *chlorella vulgaris* were determined in samples of lake water containing this pollutant. Media were prepared with filtered (0.45 μm) lake water and sterilized before use to eliminate sorption and degradation of nonalgal particulate matter (e.g., bacteria). The background nutrient concentrations of the media were 2.63 mg/l for total nitrogen, 0.09 mg/l for total phosphorus, and 29.0 mg/l for dissolved organic carbon (DOC). The initial concentration of DBP was 0.273 mg/l. The experimental conditions were the same as those of the cultural medium.

40 mL of algal suspension was centrifuged at 4000 rpm for 5 min. The supernatant was extracted with 3 mL hexane three times, and the hexane solution was analyzed with GC-FID. Algae remaining at the bottom of the centrifuging tube after centrifugation was removed by 3 mL distilled water into a histoid grinding tube, then extracted with 4 mL dichloromethane at 1400 rpm for 10 min. The solution was centrifuged at 4000 rpm for 5 min. Removing the water phase, DBP-dichloromethane solution was determined with GC-FID. Recovery of DBP from spiked water samples at a level of 50 μg/l and algal samples at a level of 50 μg/g averaged 90.9 ± 3.7% and 84.1 ± 7.2%, respectively.

DBP concentrations in water phase and in algae phase were determined by an Agilent 6890N gas chromatograph fitted with a splitless injector, a fused-silica capillary column (HP-5, 0.32 × 30 m) and a flame ionization detector. The temperature of injector and detector both were set at 250 ^{0}C. Nitrogen (50 mL/min)

was served as carrier gas; using hydrogen and airflow rates of 37 and 550 mL/min, respectively. Injection volume: 1μL. DBP was eluted with the following temperature program: 120 ℃ (2 min)→15 ℃/min (8.7min)→250℃ (3min).

Chlorophyll a was analyzed using a UV/Vis spectrometry method of Crank (1975). DOC samples were determined on a Shimadzu model VCPH total organic carbon analyzer. Nutrient determinations were made according to standard methods (National Environmental Protection Bureau 1997).

RESULTS AND DISCUSSION

Growth curves of *Chlorella vulgaris* under different conditions are shown in Fig.1. The growth process of algae in a cultural medium during 6-day period underwent a lag growth phase and an exponential growth phase. Because of different toxicities at different DBP concentrations, the algal growth rate decreased with increasing initial DBP concentration. The algal growth of 4.85 mg/L of DBP was more strongly inhibited and its exponential growth phase postponed to 96 h. For DBP experiment at 13℃, the lag growth phase lasted to the end of 6 d, due to the significant effect of low temperature on algal growth.

The growth process of algae in lake water during 6-day period had 3 phases: a lag growth phase, an exponential growth phase and a stationary growth phase. Initial lag period in lake water decreased in comparison with that in cultural medium. A decline of chlorophyll a concentration after the first 2-day increase revealed the death of cells (Fig. 2). This suggested that low nutrient level in lake water limited the growth of algae. Growth rate constants derived from density of algal cells of the culture solution under different conditions are showed in Table 1.

The logarithm of DBP concentration in algal solution vs time was plotted (Fig. 3). Good linear correlation was obtained (correlation coefficients > 0.8585), assuming that decrease rates of DBP are first order with respect to DBP concentrations. Therefore, removal rates of DBP from the algal solution were calculated with equation 1, where k_{Con}, k_{Bio} and k_{Total} are apparent first-order rate constants for control test, algal biodegradation and the sum of both of them, respectively.

$$d[DBP]/dt = -k_{Total}[DBP] = -(k_{Con} + k_{Bio})\,[DBP] \qquad (1)$$

From the control test, the loss of DBP at different experimental conditions was 4.01% ~ 4.34%, respectively, over 6 day. Therefore, it can be considered that the decline of DBP concentration in algal solution is mainly due to algal biodegradation (Fig. 4). Biodegradation first-order rate constants of DBP in *Chlorella vulgaris* are shown in Table 1.

As shown in Figure 5, with the increase of initial DBP concentration, the amounts of DBP accumulated by algae reach the maxima at 1 h for 0.317, 2.34 and 4.85 mg/L DBP, respectively, and rapidly decrease before 48 h, and then slowly decrease. Bioconcentration factors (BCFs), which represent the proportionality of

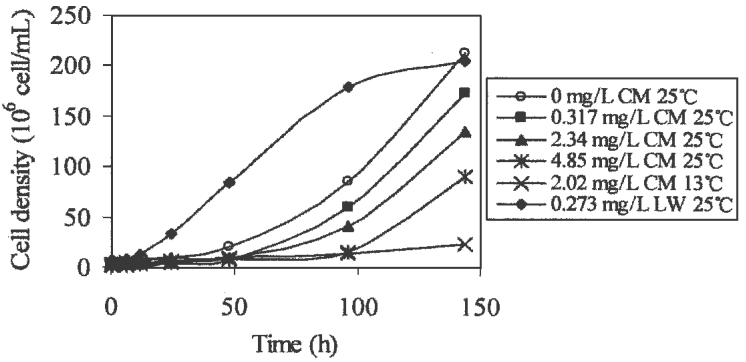

Figure 1. Growth curves of *Chlorella vulgaris* of different conditions. LW = lake water, CM = cultural medium.

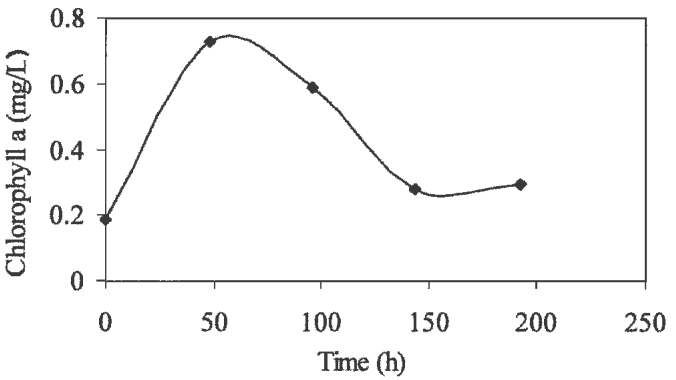

Figure 2. Chlorophyll a concentrations vs time in lake water.

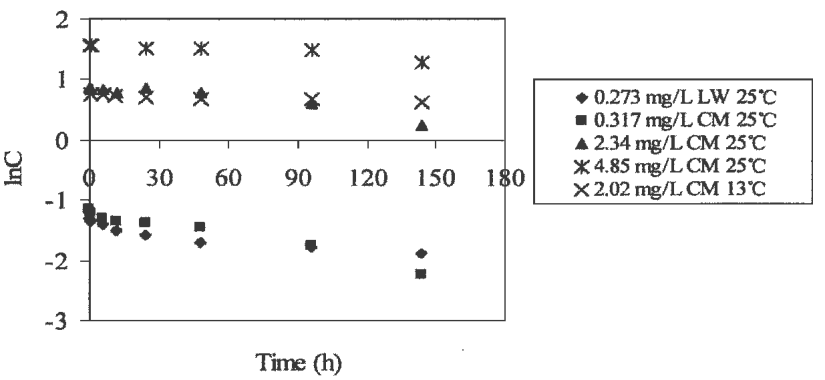

Figure 3. The logarithm of DBP concentration in algal solution vs time.

Table 1. The average growth rates (v) of *Chlorella vulgaris* and biodegradation first-order rate constants (k) of DBP in *Chlorella vulgaris* at different conditions.

T (℃)	Medium	C (mg L^{-1})	v (h^{-1})	k (10^{-3} h^{-1})
25	LW	0.273	0.034	3.2
	CM	0.317	0.037	6.8
	CM	2.34	0.029	3.6
	CM	4.85	0.022	1.3
13	CM	2.02	0.004	0.60

DBP concentration between algae and water, reach the maxima of 1.87×10^4, 5.95×10^3, and 4.46×10^3 at 1 h, respectively (Fig. 6). The change trend of BCF is very similar with that of DBP accumulation in algae.

From the experimental data obtained, the following hypothesis is suggested: There are three stages in accumulation process of *Chlorella vulgaris* after adding DBP.

The first stage is before 1h, in which the density of algal cells is small and the growth rate of algae is slow. The DBP amounts accumulated by algae and BCFs reach the maxima quickly (1h). The reason is that the surface accumulation rates of many compounds are much faster than other biological and environmental processes, their accumulation in phytoplankton cell surface can be treated as an equilibrium process (Skoglund 1996). For small algal cells, a one-compartment equilibrium model is applicable to bioaccumulation. Therefore, the DBP accumulated by algal surface diffuses into cells instantaneously. In addition, algae did not adapted to the existence of DBP in this period, which caused the accumulation rate higher than biodegradation rate.

The second stage is from 1h to 48 h, the amounts of DBP accumulated by algae and BCFs decline rapidly. In this period, the amount of algae increases no more than one magnitude, indicating that algal fission is slow, and the algae grow up mainly as a size of individual but not as biomass. The reproduction way of *Chlorella vulgaris* is asexual reproduction. The mitosis of an eukaryotic cell happens 2~3 times continuously and produces 4~8 autospores, then the eukaryotic cell splits and autospores live as individuals (Chen 1999). A eukaryotic cell before split is 5 times bigger than a single autospore. Cheng et al. (2003) reported that surface accumulation rate constants of HOCs were much higher than intracellular accumulation rate constants. The larger algal cells are, the more time it takes for the cells to reach a steady state. Therefore, with increasing algal cell volume, the cytoplasm increases, and the concentration of DBP in the interior compartment decreases in combination with the algal biodegradation, because the algae have gradually adapted to the existence of DBP.

The decrease of the concentration of DBP in the surface compartment (C_s) is also an important reason for the decline of BCF and DBP accumulated by algae. The surface component of accumulation is best quantified by a partitioning equation (Equation 1), where K_{SW} is the surface adsorption partitioning coefficient, and C_d

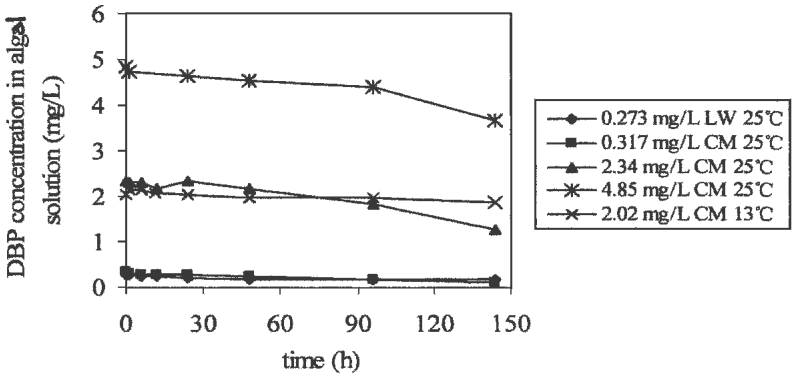

Figure 4. The declines of DBP in algal solutions under different conditions.

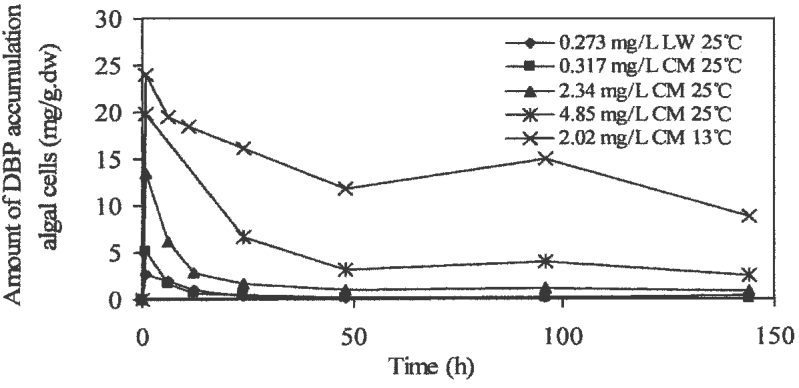

Figure 5. DBP accumulation by *Chlorella vulgaris* under different conditions.

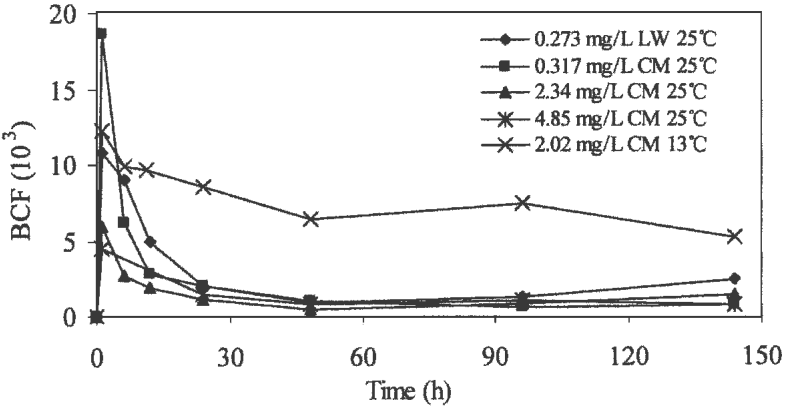

Figure 6. BCF of DBP by Chlorella vulageris under different conditions.

is the dissolved concentration of DBP:

$$C_s = C_d K_{SW} \quad (2)$$

The total decreases of DBP in algal solution include three aspects: biodegradation by algae, photolysis, and volatilization, all of which followed first-order kinetics. For the first-order kinetic process, the decrease of DBP is much greater at prophase, which causes the thermodynamic reallocation of DBP between algae and water, resulting in a significant decline of BCF and DBP accumulation.

The third stage is after 48 h, the amounts of DBP accumulated by algae and BCF decline gradually. This period belongs to an exponential growth phase, the algal growth rate become larger, and the amount of algal cells increases two magnitudes more than that of initial algal cells. Because of the rapid fission of cells, the total amounts of DBP in cells decreased with biodilution (Skoglund 1996) that makes the accumulation amounts of DBP and BCF decreased.

The effects of DOC on HOC bioavailability in phytoplankton have been studied with various concentrations of humic acid (Twiss 1999), peat moss infusion (Richer 1993), and cell exudates (Sijm 1995). The results of these studies indicate that the bioavailability of HOCs is reduced by DOC. During exponential growth phase, with the rapidly increasing alga growth rate, the cell exudates increase fast accordingly. As a result, BCF and the accumulation amounts of DBP decrease.

The first-order rate constant values of DBP increased from 1.3×10^{-3} to 6.8×10^{-3} h^{-1} as decreases in DBP concentrations from 4.85 to 0.317 mg L^{-1} (Table 1). This might be due to increasing toxicity of DBP with the increase of DBP concentration (Fig. 1), which inhibits the biodegradation activity.

As shown in Figures 5 and 6, accumulation amounts of DBP by algae and BCFs at 13℃ and 25℃ at similar initial DBP concentration both reach the maxima at 1 h. The accumulation amounts of DBP by algae and maximum of BCF (1.23×10^4) at 13℃ was higher than that at 25℃. The temperature effect may be partly explained as an effect on DBP solubilities, as increasing temperature leads to higher aqueous solubilities and thus lower water-organic matter partitioning coefficients (Chen 1999; Schwarzenbach 2003). The higher degradation rate of DBP by algae at 25℃ can also reduce the accumulation amounts of DBP by algae more significantly, and lower the BCF.

The first-order degradation rate constant of DBP by algae at 25℃ is higher than that at 13℃ (Table 1). Studies have showed that biodegradation rate of PAEs is influenced by temperature and longer half-life is likely at low temperature (Staples 1997). Biodegradation rate of DEHP at temperature 10 °C was found to be less than 50% of that of DEHP at temperature 18 °C (Peijnenburg 1991). Therefore, phenomenon in this study is believed to be due to inhibition of biodegradation activity at low temperature.

The change trend of accumulation amounts of DBP by algae and BCFs is very similar with that in the cultural medium (Figs. 5 and 6). The accumulation amount of DBP by algae and BCF in lake water both reach the maxima at 1 h. The maximum of BCF is 1.08×10^4 in lake water, lower than in the cultural medium. The lipid content of algae is known to be the major factor influencing the bioconcentration of HOCs (Manthey 1993) and algal lipid status may vary considerably for the same species in relation to nutritional status. In general, nitrogen-deficient microalgae have a higher proportion of lipid (Miller 1962). For this investigation, the concentration ratio of total nitrogen to total phosphor was 29.2 in lake water, and the lake water was P limited. Therefore, the possible reason for BCF difference between in lake water and in the cultural medium is that difference of nutritional status causes the change of algal cell composition. The first-order degradation rate constant of DBP by algae in lake water is lower (Table 1). This might be explained as the lower accumulation amount in algal cells.

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